Emergent Vegetation Coverage and Human Activities Influence Oviposition Microhabitat Selection by Invasive Bullfrogs (*Lithobates catesbeianus*) in Southwestern China

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Xuan Liu,[1] Yisong Guo,[2] Yu Luo,[3] and Yiming Li[1,4]

[1]*Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang District, Beijing 100101, China*
[2]*Department of Ecology, Chemistry and Environmental Engineering, Yunyang Teachers' College, Danjiangkou 442000, China*
[3]*College of Life Science, Guizhou Normal University, Guiyang 550001, China*

ABSTRACT.—Oviposition site selection is critical to increase offspring fitness for aquatic-breeding anurans, which generally lack posthatching parental care. Understanding factors that influence oviposition microhabitat choice would be helpful for managing and controlling invasive amphibians, such as the American Bullfrog (*Lithobates catesbeianus*), implicated in the decline of anuran populations across its invasive range. Factors that influence bullfrog oviposition microhabitat selection, however, remain mostly unknown. On the basis of field surveys from 2008 to 2013, we investigated 46 oviposition sites used by bullfrogs and 46 adjacent random unused sites and evaluated eight characteristics that could potentially influence oviposition site selection by bullfrogs in Caohai wetland, southwestern China, a global biodiversity conservation hot spot. We used model averaging of generalized linear mixed-effects models, using Akaike information criterion, hierarchical partitioning, and an ordination principal components analysis to analyze site selection. Results revealed that bullfrogs preferred waters with a high proportion of emergent plant coverage, whereas the distance to the nearest boat route decreased the probability of bullfrog oviposition site presence. Our study was one of the first quantitative studies on oviposition microhabitat selection by bullfrogs within their invaded ranges that combined abiotic, biotic, and human activities. We suggest that efforts to control and manage this global invader be directed toward reducing the suitability of breeding microhabitats by targeting areas with dense emergent vegetation, far from human disturbances.

Amphibian declines and extinctions represent a critical conservation concern around the world (Stuart et al., 2004). Alien species invasions have been regarded as an important causal factor in the decline of amphibians (Kats and Ferrer, 2003). Among these, the American Bullfrog (*Lithobates catesbeianus*; "bullfrog" hereafter) is a representative species. Native to southern Quebec in Canada and the southern and eastern United States, bullfrogs have broadly invaded over 50 regions and countries worldwide (Kraus, 2009), and is one of the "100 of the world's worst invasive species" (Lowe et al., 2004). In their invaded ranges, bullfrogs may have multiple negative impacts on native amphibians by predation (Wu et al., 2005; Silva et al., 2011), competition (Kupferberg, 1997; Kiesecker et al., 2001), reproductive interference (Pearl et al., 2005; D'Amore et al., 2006), and vectors of amphibian diseases (e.g., chytrid fungus [*Batrachochytrium dendrobatidis*, "*Bd*"]; Garner et al., 2006; Liu et al., 2013b). Overall, bullfrogs have been implicated in population declines and local extinctions of native amphibians in many parts of the world where they have invaded (Moyle, 1973; Bury and Luckenbach, 1976; Fisher and Shaffer, 1996; Li et al., 2011).

Managing and controlling invasive species is considered very difficult once they have established feral populations (Simberloff, 2003). This is especially true for bullfrogs (Govindarajulu et al., 2005; Adams and Pearl, 2007), as they have a high capacity for rapid adaptability to novel environments (Liu et al., 2010; Li et al., 2014), rapid range expansions that have been evident from population genetic data (Austin et al., 2003), large-scale spatial–temporal analyses in invaded ranges (Liu et al., 2014), and superior fecundity observed in both their native (Howard, 1978; Bruneau and Magnin, 1980; Bury and Whelan, 1985) and invasive ranges (Govindarajulu et al., 2006; Kaefer et al., 2007). For example, in their native range, each female bullfrog can produce

more than 20,000 eggs (Howard, 1978; Bruneau and Magnin, 1980; Bury and Whelan, 1985). In their invaded regions, clutch sizes have reached 13,014 eggs in British Columbia, Canada (Govindarajulu et al., 2006) and 26,200 eggs in southern Brazil (Kaefer et al., 2007). Previous studies have suggested multiple means to control this highly invasive species through direct removal (Banks et al., 2000) and habitat modifications, such as hydrological alterations for pond drying (Maret et al., 2006). Unfortunately, these approaches largely focused on adult individuals or tadpoles and were strongly limited by landscape characteristics (Doubledee et al., 2003; Govindarajulu et al., 2005).

Bullfrogs were first introduced into China in the 1980s as a food source and then expanded to many provinces (Liu and Li, 2009). They have since established feral populations across the country from eastern islands to southwestern plateau areas through escapees from aquaculture enclosures and human release (Li et al., 2006; Liu and Li, 2009; Liu et al., 2012, 2013a). In the Zhoushan archipelago, field surveys showed that native frog densities and species richness were negatively related to invasive post-metamorphic bullfrog densities (Li et al., 2011). Diet composition analysis and control experiments also verified that bullfrogs in the Zhoushan archipelago could predate upon native frogs (Wu et al., 2005; Wang et al., 2007). This species has long been postulated to be the cause of the extinction of the endemic Yunnan Lake Newt (*Cynops wolterstorffi*) in Kunming Lake (He 1998) and Vocal-Sacless Spiny Frog (*Paa liui*) in Lugu Lake (Li and Xie, 2004). A recent quantitative study confirmed that invasive bullfrogs in Coahai wetland, Lugu Lake prefer to consume endemic Yunnan pond frogs (Liu et al. 2015). In addition, *Bd* has been detected in bullfrogs and native frogs in both feral populations (Bai et al., 2012) and museum specimens (Zhu et al., 2014a) across China. Recently, another chytrid species, *Batrachochytrium salamandrivorans*, was isolated in the Netherlands, although it has not been detected in
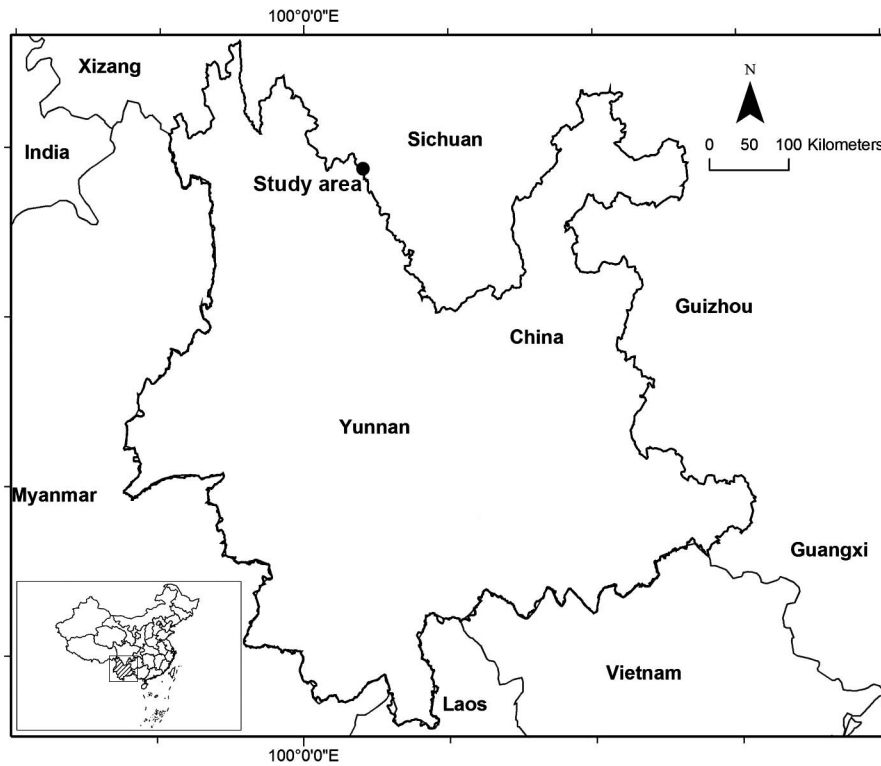
Fɪɢ. 1.   Map of the study area showing the bullfrog oviposition field survey site in Caohai wetland, Lugu Lake, southwestern China.

the invasive bullfrogs in China (Zhu et al., 2014b). Therefore, biologists urgently need effective prevention and management strategies to control further bullfrog invasions.

Suitable oviposition sites are particularly important for species that generally lack maternal care after oviposition (e.g., amphibians; Refsnider and Janzen, 2010). Oviposition site selection influences hatching success and larval growth as water physical conditions, vegetation, co-occurring species, and human activities could influence the suitability as an oviposition site (Resetarits and Wilbur, 1989). Theoretically, amphibians move least at the egg stage; consequently, identifying where bullfrogs prefer to oviposit may provide an excellent opportunity to develop specific strategies to eliminate propagule pressures by directly removing their eggs. Adult bullfrogs are primarily an aquatic species that prefer permanent still waters (Bury and Whelan, 1985; Wang and Li, 2009), because bullfrog tadpoles generally overwinter and require >2 yr before metamorphosis (Willis et al., 1956). Within permanent waters, bullfrogs prefer to lay eggs in deeper areas with dense vegetation coverage (Cook and Jennings 2007), but we found no quantitative studies on bullfrog oviposition microhabitat selection that combined abiotic and biotic factors with human activities. Therefore, because of the amount of human activity at Lugu Lake, we conducted the first quantitative study that simultaneously evaluated the potential biotic, abiotic, and human impact factors and their independent relative contributions to bullfrog oviposition microhabitat selection in Caohai wetland, a part of Lugu Lake in southwestern China. Caohai, located at Zuosuo town at the border of Ninglang County of Yunnan Province and Yanyuan County of Sichuan Province (Fig. 1), is a grassy plateau contiguous wetland with permanent still waters <6 m deep and an area of approximately 7 km². It is a part of Lugu Lake, the highest-altitude and the second deepest freshwater lake in Yunnan Province. Caohai has a typical plateau monsoon climate with an annual average temperature of 17°C,

abundant rainfall, and clearly defined wet and dry seasons. Bullfrogs were introduced into Caohai in the 1980s and have had established feral populations here for over 20 yr (Liu et al., 2010). In addition to introduced bullfrogs, historical literature shows the presence of several native amphibian species such as the Yunnan Pond Frog (*Babina pleuraden*), the Large-Webbed Bell Toad (*Bombina maxima*), the Yunnan Odorous Frog (*Odorrana andersonii*), and the Vocal-Sacless Spiny Frog (*P. liui*) (Yang et al., 1991; Fei, 1999); during our field surveys in recent years, we did not record the presence of *O. andersonii* and *P. liui* but only *Babina pleuraden* and *Bombina maxima* (Liu and Li, 2009; Liu et al., 2012, 2013a).

MATERIALS AND METHODS

Field Surveys.—We used data from a 5-yr field study (2008–2013) in Caohai wetland, Lugu Lake. During the breeding season from June to August 2008 to 2013, we carefully searched for evidence of bullfrog egg clutches and recorded the bullfrog oviposition habitats on rainless days between 0500 h and 1700 h. Because we conducted a systematic survey, we standardized our survey efforts across the accessible part of Caohai wetland. We recorded the location of each site and collected biotic, abiotic, and human activity factors that might affect the choice of bullfrog oviposition site selection. At each oviposition site, we centered a 1 m × 1 m grid over the egg clutch and measured the water pH, water temperature (°C), water depth (m), and distance (m) to the nearest boat route (Howard, 1978; Xu and Li, 2013). We also used dip nets to record the number of potential predators including odonate naiads and fish, predators of bullfrogs in the United States (Adams et al., 2003; Smith et al., 2008). In addition, we divided the grid into 100 subgrids and estimated the percentage of water surface covered by emergent and floating plants with an accuracy of 1%. After the oviposition site surveys, we repeated

TABLE 1. Comparison of habitat characteristics between the surveyed oviposition sites and random sites in Caohai wetland, Lugu Lake, China. Values are means or proportions ± SE.

Characteristics	Oviposition site	Random site	Test[a]
Water temp. (°C)	24.25 ± 0.36	24.91 ± 0.22	1.16
Water pH	8.08 ± 0.06	7.97 ± 0.07	1.37
Water depth (m)	0.81 ± 0.04	0.72 ± 0.04	2.10
Emerg. veg. cover (%)	71.74 ± 3.47	30.33 ± 3.16	41.57**
Floating veg. cover (%)	58.26 ± 4.09	54.57 ± 4.38	0.34
Number of fish	1.43 ± 0.28	1.50 ± 0.29	0.02
Number of odonates	1.63 ± 0.21	1.83 ± 0.25	0.14
Dist. nearest boat route (km)	0.43 ± 0.05	0.19 ± 0.03	14.19**

[a] χ^2 values from a Kruskal–Wallis test.
** $P < 0.001$.

these habitat measurements at random polar coordinates located within 50 m of each oviposition site (Xu and Li, 2013). We then returned to each oviposition site and collected the entire egg clutch in a plastic bag with water for later laboratory analysis. We randomly selected 2,000 eggs from each clutch and measured the diameter of each egg to the nearest 0.02 mm using a Vernier caliper.

Statistical Analyses.—We first compared differences in habitat characteristics between bullfrog oviposition sites and random sites with Kruskal–Wallis tests. We then used generalized linear mixed-effects models (GLMMs) with a logit link and binomial error distribution to investigate the effects of eight predictor variables on bullfrog oviposition site selection (Table 1; oviposition site = 1, random site = 0). To account for the variation in temporal frequency of oviposition site locations across the 5 yr of this study, sampling time was entered as a nested random effect; all other variables were treated as fixed effects. For the two biotic factors, we also used the presence of odonate naiads and fish as independent variables, considering that numbers of certain species might vary across space and time during 1 d. We created models using all possible combinations of the eight variables (total 255 models: $2^8 - 1$), and used a model-averaging approach to estimate the effect of each variable (Burnham and Anderson, 2002). This approach is regarded as robust by using an entire set of models to make a more reliable inference than selecting a single best model (Whittingham et al., 2006). We calculated the relative importance of each variable by summing Akaike weights across all GLMMs based on the Akaike information criterion (Burnham and Anderson, 2002). Furthermore, because of collinearity among variables (Table S1), we applied hierarchical

partition analysis to evaluate the unique and shared variance of each variable and determined the statistical significance of each variable's unique variance using randomization tests ($n = 100$) based on an upper confidence limit of 0.95 (a pseudo-Z score > 1.65; Mac Nally, 2002). Finally, considering that more nuanced patterns among variables would potentially be excluded because of the limitations associated with regression analysis, we used an ordination principal components analysis (PCA) to better visualize the relative importance of particular variables in explaining the differences between oviposition sites and random sites in a two-dimentional space. We conducted all analyses in R (Version 2.15.1, R Development Core Team, 2012); we used the "lmer" function in the "lme4" package to perform GLMMs analysis and used the "dredge" and "model.avg" functions in the "MuMIn" package to perform the model-averaging analysis. We performed the hierarchical partitioning analyses using the "hier.part" and "rand.hp" functions in the hier.part package. We used "dudi.pca" function in the "ade4" package to perform PCA. All descriptive statistics are reported as means (± SE).

RESULTS

We found 10, 6, 7, 8, and 15 oviposition sites in 2008, 2009, 2011, 2012, and 2013, respectively, and selected the same number of random sites each year. Mean clutch size was 10,500 ± 930.65 eggs, and mean diameter of randomly sampled eggs was 1.46 ± 0.003 mm.

The Kruskal–Wallis test showed a greater percent cover of emergent plants in the oviposition sites than in random sites (Table 1). In addition, the distance of oviposition sites to the nearest boat route was farther than that for random sites (Table 1); however, there were no significant differences in the other factors between oviposition and random sites (Table 1).

Model averaging suggested that emergent vegetation cover and the distance to the nearest boat route were the two most important variables to the bullfrog oviposition site selection (Table 2). We found a positive effect of mean percent cover of emergent vegetation at oviposition sites and a negative relationship between oviposition site and distance to the nearest boat route (Table 2). The hierarchical partition analysis confirmed the model-averaging results and showed that mean percent cover of emergent plant and the distance to the nearest boat route were the two variables that explained the largest independent variations with a statistically significant effect on bullfrog oviposition site selection (Table 2). These two variables explained >92% of the total deviation (Table 2). PCA analyses also showed them to be the two most important variables along the first

TABLE 2. A summary of model-averaging and hierarchical partitioning analyses based on the GLMMs with oviposition site selection (i.e., oviposition site = 1, random site = 0) as the response variable and eight abiotic, biotic, and human activity factors as the explanatory variables, sampling time as nested random effect. Model-averaged 95% confidence intervals that do not overlap zero are shown in bold.

Explanatory variables	Model averaging			Hierarchical partitioning	
	Relative importance	Parameter estimate	Variance	Independent contribution	Independent deviation explained (%)
Water temp. (°C)	0.29	0.11	0.16	0.54	1.83
Water pH	0.24	−0.11	0.70	0.28	0.93
Water depth (m)	0.25	−0.23	1.08	0.50	1.67
Emerg. veg. cover (%)	**1.00**	**6.72**	1.41	22.70	76.37**
Floating veg. cover (%)	0.37	−1.47	1.41	0.55	1.85
Number of fish	0.29	−0.12	0.17	0.18	0.60
Number of odonates	0.26	−0.08	0.21	0.11	0.36
Dist. nearest boat route (km)	**0.87**	**2.56**	1.14	4.87	16.39**

** Statistically significant ($P \geq 0.05$) estimates of unique deviation determined using randomization tests based on 95% confidence intervals (pseudo-Z score > 1.65).
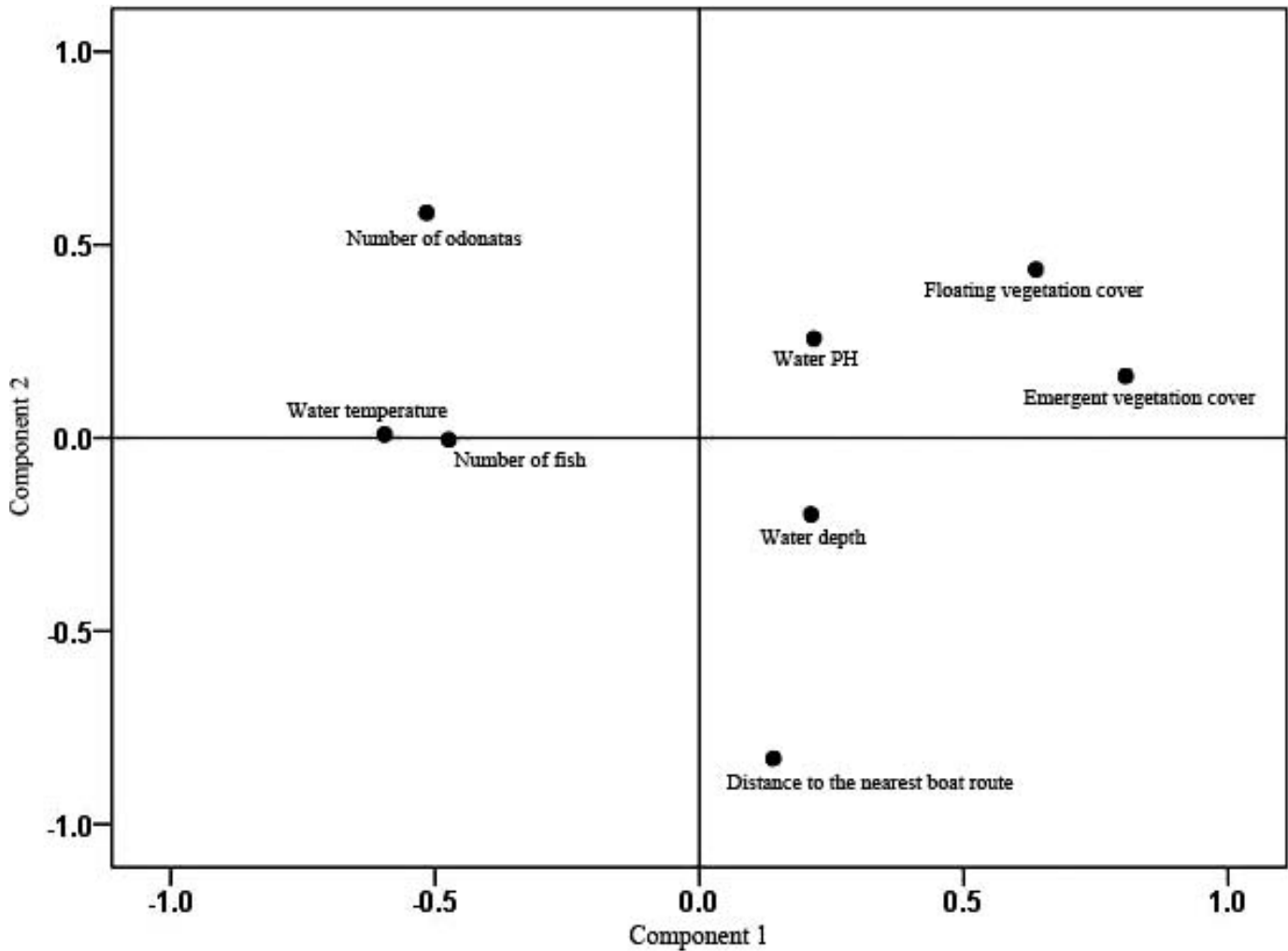
FIG. 2. Factor map of the principal components analysis (PCA) showing the relative importance of different microhabitat variables in explaining the bullfrog oviposition site selection in a two-dimensional space.

component and the second component, respectively (Fig. 2). We obtained similar results when we used the presence of odonate naiads and fish, rather than their abundance (Table S2).

DISCUSSION

This was the first quantitative study on oviposition microhabitat selection by bullfrogs that combined abiotic, biotic, and human activities in their invaded ranges. We also provided new evidence of the high reproductive capacity of this species in our study area compared with that in their native and invaded ranges. Physical water characteristics and biotic factors (e.g., presence and abundance of potential predators) did not appear to be related to bullfrog oviposition microhabitat choice; instead, we found that emergent plant coverage and distance to the nearest boat route to be the two most important variables: bullfrogs showed a preference for oviposition sites with more emergent plant coverage that were farther from boat routes.

As amphibians generally have no parental care, our findings supported the hypothesis that reducing the predation risk might be a primary factor in oviposition site choice for amphibians (see review in Refsnider and Janzen, 2010). Mating at sites with a high density of emergent plants would effectively hide adults in amplexus from predators and might simultaneously increase embryo survival. This was consistent with findings that

vegetation could reduce the predation efficiency of natural enemies on bullfrogs in native ranges (Howard, 1978). This also supported findings on the important role of vegetation coverage in bullfrog oviposition site selection in California (Cook and Jennings 2007). In addition, as our study was located at an average elevation of 2,800 m, the high coverage of emergent plants also could decrease ultraviolet radiation exposure of eggs (Palen et al., 2005). Previous studies found that human activities were negatively related to the establishment of feral populations (Li et al., 2006; Ficetola et al., 2007). The relatively important role of distance to boat routes on the selection of bullfrog oviposition sites demonstrates that human disturbances along boat routes also affected their oviposition microhabitat selection. This result is not surprising, as human activities (e.g., fishing and tourist vessels) would be most frequent along boat routes and likely would have negative impacts on bullfrog breeding.

Although odonate naiads and fish represent predatory species of bullfrog tadpoles in the United States (Adams et al., 2003; Smith et al., 2008), we detected no direct effects in both the presence or abundance of odonate naiads and fish on bullfrog oviposition site choice in our study area. One potential explanation is that predators might have different impacts on bullfrog eggs and tadpoles, as previous studies suggest that the thick jelly surrounding developing embryos of *Rana* species has

a protective function against predation by many invertebrates and fish (Jennings and Schaefer, 1978; Henrikson, 1990). Alternatively, because bullfrogs generally lack coevolved enemies in their invaded ranges, the enemy release hypothesis (Shea and Chesson, 2002) also might facilitate their invasion success. In Michigan, leeches (*Macrobella decora*) were one of the major predators on anuran embryos, including bullfrogs (Howard, 1978). Nevertheless, we did not record this species or similar leech species in our study area during our field surveys. A similar situation occurs for bullfrog tadpoles. Matsushima and Kawata (2005) suggested that amphibians tend not to oviposit in waters containing conspecific larvae to avoid cannibalistic and intraspecific competition. We did not include the presence of bullfrog tadpoles in our analysis, because bullfrog tadpoles occupied most of the oviposition sites (91.7%) and random sites (93.1%); therefore, we could not obtain robust statistical power of this variable. Our results were similar to those for Cane Toads (*Bufo marinus*) in Australia, where a field correlative study failed to find a relationship between toad oviposition and the presence of other predator or competitor organisms (Hagman and Shine, 2006). We recommend using field mesocosm experiments to further investigate the more complex interactions between bullfrogs and the presence, abundance, and richness of other aquatic competitors and predators.

Thermal conditions also are considered to be important in accelerating and maximizing embryo development and survival in amphibians, especially for pond-inhabiting frog species that usually choose deep and warm sites for oviposition (Petranka and Petranka, 1981; Bull and Shepherd, 2009). In their native ranges, bullfrogs avoided areas with high water temperatures (>32°C) that would result in developmental abnormalities (Howard, 1978). Nevertheless, the water temperatures we measured in both breeding and random sites were within the 32°C threshold, and may explain why we did not find a significant effect of water temperature on the presence of bullfrog eggs. In our high-altitude study area, adult bullfrogs have exhibited decreased body size and sexual size dimorphism compared with a low-altitude population (Liu et al., 2010); whether this truly is an adaptation to cool temperatures or just phenotypic plasticity still is not clear. Future research will be required to detect whether the traits at early egg stage also have exhibited similar geographical variations, which might be helpful to determine the mechanism of this variation and provide more information on bullfrog strategies for successful invasion.

This study may have important conservation implications for the control of invasive bullfrog populations. Future strategies can consider reducing the propagule pressure at the breeding stage to minimize their reproductive success; this would include searching for bullfrog oviposition sites in areas with dense emergent plant cover, far from human activities to eradicate bullfrog eggs; however, once the oviposition site was found and eggs were removed, adult bullfrogs might disperse quickly to more recipient habitats, and intensive monitoring would be needed to prevent their further spread. In addition, hunting for bullfrog eggs may potentially and inadvertently destroy suitable habitats for native species. For example, the endemic *Babina pleuraden* has a sympatric distribution with the bullfrog in the study area (Liu and Li, 2009). Interestingly, we recorded only two clutches of *B. pleuraden* during our field surveys. The native species may have imposed adaptations, such as the separation of oviposition sites across space and time, to reduce the impact of the bullfrog; this also warrants future studies.

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